EXHIBIT F

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Biochemistry

Involvement of *ras* p21 protein in signal-transduction pathways from interleukin 2, interleukin 3, and granulocyte/macrophage colony-stimulating factor, but not from interleukin 4

(growth factor receptor/lymphokine/cytokine)

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ABSTRACT **The protooncogene *ras* acts as a component of signal-transduction networks in many kinds of cells. The *ras* gene product (p21) is a GTP-binding protein, and the activity of the protein is regulated by bound GDP/GTP. Recent studies have shown that a certain class of growth factors stimulates the formation of active p21·GTP complexes in fibroblasts and that oncogene products with enhanced tyrosine kinase activities have a similar effect on *ras* p21. We have measured the ratio of active GTP-bound p21 to total p21 in several lymphoid and myeloid cell lines in order to understand the role of *ras* in the proliferation of these cells. Interleukin 2 (IL-2), IL-3, and granulocyte/macrophage colony-stimulating factor (GM-CSF) enhance the formation of the active p21·GTP, whereas IL-4 has no effect on p21-bound GDP/GTP. These results strongly suggest that *ras* p21 acts as a transducer of signals from IL-2, IL-3, and GM-CSF, but not from IL-4.**

Various kinds of growth factors trigger proliferation of cells when they bind to their specific receptors. Receptors complexed with the ligands input signals through the plasma membrane, and intracellular signals from growth factor receptors are transmitted through a large number of signal-transduction pathways. In many cases, growth factor receptors themselves have tyrosine kinase activity, or they are associated with other tyrosine kinases, and phosphorylation of tyrosine residues on the target molecules is essential for activation of the pathways. The *ras* proteins (p21) were originally identified as oncogene products (1). They belong to a family of signal-transducing monomeric GTP-binding proteins, which are thought to play an essential role in transducing and amplifying growth signals inside the plasma membrane.

Since GTP-bound p21 is an active form in transducing signals, we have quantitated the activity of p21 in cells by measuring the composition of bound GDP/GTP (2, 3). This method was successfully applied to fibroblast cell lines and it was confirmed that some definite growth factor receptors with tyrosine kinase activity [for example, the platelet-derived growth factor (PDGF) receptor and the epidermal growth factor (EGF) receptor] transduce signals for the initiation of DNA synthesis through activation of *ras* p21 (4–6). Oncogene products with constitutively enhanced tyrosine kinase activities, including both receptor and non-receptor proteins, were found to increase the amounts of the activated GTP-bound p21 when they transformed cells (5, 6). These observations provided direct evidence for biochemical linkage between tyrosine kinases and *ras* p21. It has been reported that stimulation of the T-cell receptor with phytohemagglutinin or anti-CD3 monoclonal antibody caused the activation of *ras* p21 (7). In this case, the involvement of tyrosine kinases in signal transduction is not known.

A variety of cytokines are involved in survival, proliferation, and differentiation of hematopoietic cells. Recently, cDNAs of various cytokine receptors were cloned, and it has been revealed that cytokine receptors including those for interleukin 2 (IL-2), IL-3, IL-4, and granulocyte/macrophage colony-stimulating factor (GM-CSF) are members of a receptor family that are distinct from growth factor receptors with a tyrosine kinase and from hormonal receptors coupled with a heterotrimeric GTP-binding protein (G protein) (8). These cytokine receptors have no intrinsic tyrosine kinase, and high-affinity binding of IL-2, IL-3, and GM-CSF to their receptors involves at least two receptor subunits. Networks of intracellular signaling in lymphoid and myeloid cells from cytokine receptors also seem to be complicated and remain unclear. Since we have established, in the course of the recent studies, a useful procedure to quantitate the activity of *ras* p21 in living cells, we have expanded our interests to the role of *ras* in signal transduction systems from cytokines and made an attempt to analyze the activity of *ras* p21 in response to various cytokines in cultured lymphocyte and myeloid cell lines.

In this paper, we report the involvement of *ras* p21 in signal transduction from IL-2, IL-3, and GM-CSF, but not from IL-4. Increases in GTP-bound active *ras* p21 were observed in several types of cultured lymphocytes and myeloid cell lines after the addition of IL-2, IL-3, or GM-CSF. Since these growth factors are strictly required for the survival and growth of these cells, the results strongly support the idea that *ras* p21 transduces signals controlling cell growth in lymphocytes and myeloid cells. On the other hand, IL-4 seems not to transmit signals through *ras* p21, though IL-4 can act as an indispensable factor for survival or growth of these cell lines.

MATERIALS AND METHODS

Materials. Purified recombinant mouse IL-2 and IL-4 were kindly provided by Gerard Zurawski and Nobuyuki Harada (DNAX Research Institute), respectively. Mouse IL-3 and GM-CSF were obtained as described (9, 10). EGF, phorbol 12-myristate 13-acetate (PMA), and ionomycin were purchased from Takara Shuzo (Kyoto), Sigma, and Calbiochem, respectively.

Cell Culture. The cell lines were cultured in RPMI 1640 supplemented with 10% (vol/vol) fetal bovine serum and one of the following lymphokines: IL-2, IL-3, IL-4, or GM-CSF. The cells were precultured in the presence of the same

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Abbreviations: IL-*n*, interleukin *n*; GM-CSF, granulocyte/macrophage colony-stimulating factor; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; PMA, phorbol 12-myristate 13-acetate; GAP, GTPase-activating protein.

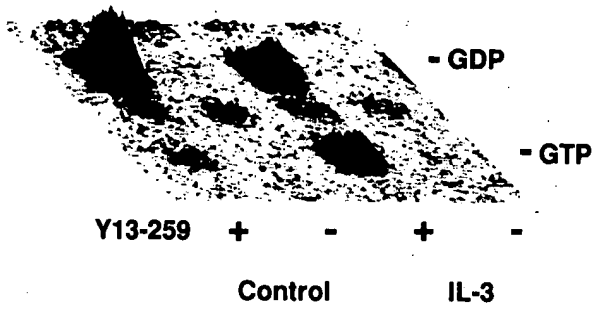

FIG. 1. Accumulation of p21·GTP in IC2-E cells treated with IL-3. The p21-bound GDP and GTP were analyzed after the addition of IL-3 (5 ng/ml) or control buffer for 10 min. The TLC plate was scanned with an AMBIS radioanalytic system, and the intensity of radioactivities is shown as three-dimensional graphics. Y13-259 is a monoclonal antibody against *ras* p21.

lymphokine to be tested for the effect on p21 activation except that HT-2, BAF3, and IC2 cells tested for the effect of IL-4 were cultured in IL-2-, IL-3-, and IL-3-containing media, respectively. IC2-E cells were cultured in IL-3-containing medium supplemented with G418 (0.8 mg/ml) in all assays.

Analysis of *ras* p21-bound GDP/GTP. Cells were seeded at a density of 4.6×10^3 per ml (for IC2-E cells) or 1.5×10^3 per ml (for the other cells). After 3 days of culture, the cells were collected and washed with phosphate-free RPMI 1640 once. Then, the cells were suspended and incubated in phosphate-free RPMI 1640 containing [^{32}P]orthophosphate (0.4 mCi/ml, carrier-free, NEX-053, NEN; 1 mCi = 37 MBq) for 150 min. During this period, the cells were arrested at the quiescent state by deprivation of both fetal bovine serum and any lymphokines and simultaneously were labeled with ^{32}P. A lymphokine or control buffer was then added to the cells for a specified period. The cells were washed and disrupted under mild conditions and the analysis of p21-bound GDP/GTP was carried out as described (4).

RESULTS

Analysis of p21-bound GDP/GTP in Myeloid Cell Lines. From previous studies (4–6) it has become apparent that various types of tyrosine kinase can activate *ras* p21 when stimulated by growth factors or mutated to induce transformation. In this study, we analyzed the effects of various cytokines on the activity of *ras* p21 in lymphoid and myeloid cells by measuring the p21-bound GDP/GTP. p21 was immunoprecipitated from ^{32}P-labeled cells with anti-p21 monoclonal antibody Y13-259 and analyzed by thin-layer chromatography as described previously (3, 4), with some modifications.

A typical result of the analyses is shown in Fig. 1. The mouse mast cell line IC2-E grows in medium containing IL-3, and deprivation of IL-3 results in cell death within a day; i.e., IL-3 is an essential factor for survival and growth of this cell line (11). We compared p21-bound GDP/GTP between IL-3-stimulated and unstimulated IC2-E cells and found that the ratio of GTP-bound active p21 to total p21 increased within 10 min (Fig. 1). For Fig. 1, the radioactive intensity of each nucleotide on the thin-layer plate was measured directly by a radioanalytic imaging system, and the result was visualized as a three-dimensional image. The results indicate that *ras* is involved in the signal-transduction cascade from IL-3 in IC2-E cells.

IL-2, IL-3, and GM-CSF, but Not IL-4, Stimulate Formation of Active p21·GTP Complexes. We have chosen four well-characterized lymphokines—IL-2, IL-3, IL-4, and GM-CSF—and tested for the possible involvement of *ras* in the signal-transduction pathways from these factors in a number of cultured cell lines. In all cases, we have confirmed the biological action of the lymphokines on the cell lines. The criterion is either of the following: (*i*) the cells can grow in the presence of the tested growth factor or (*ii*) the tested factor can maintain the viability of the cells at least for a relatively short period. In both cases, the removal of the factor causes the rapid death of the cells.

First, we examined the effect of IL-2. IL-2 is produced by antigen-stimulated helper T cells and acts as a T-cell growth factor. We used two T-cell lines, CTLL (12) and HT-2 (13). The addition of IL-2 to these cells caused a 3- to 6-fold increase of the active p21·GTP form within 10 min (Fig. 2).

IL-3 is a lymphokine that acts on bone marrow stem cells to generate multilineage colonies. As shown in Fig. 3, accumulation of GTP-bound active p21 could be observed after the addition of IL-3 in all three IL-3-dependent cell lines: pre-B-cell line BAF3 (14) and mast cell lines IC2 (15) and PT18 (16). The fold increase of the ratio of p21·GTP to total p21 was

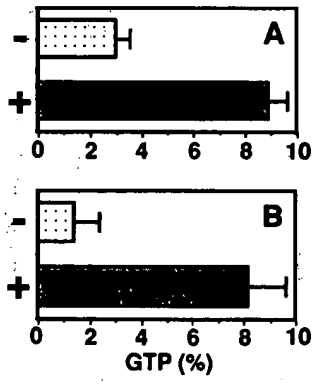

FIG. 2. Increase of p21·GTP in T cells induced by IL-2. CTLL (*A*) and HT-2 (*B*) cells were treated with IL-2 (5 ng/ml) (+) or control buffer (−) for 10 min, and the p21-bound GDP and GTP were analyzed. The ratio of p21·GTP to p21·GDP plus p21·GTP is shown as a percentage (mean ± SEM, $n = 3$ or 4).

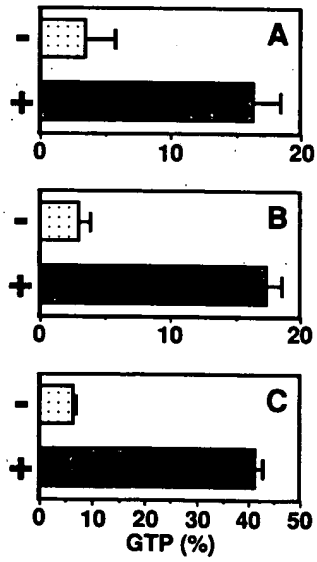

FIG. 3. Increase of p21·GTP in B cells and mast cells induced by IL-3. BAF3 (*A*), IC2 (*B*), and PT18 (*C*) cells were treated with IL-3 (5 ng/ml) (+) or control buffer (−) for 10 min, and the p21-bound GDP and GTP were analyzed. The ratio of p21·GTP to p21·GDP plus p21·GTP is shown as a percentage (mean ± SEM, $n = 3$ or 4).

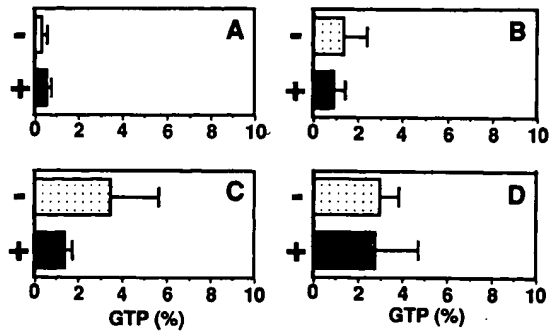

FIG. 4. IL-4 does not increase p21·GTP in T cells, B cells, and mast cells. MC9-4 (*A*), HT-2 (*B*), BAF3 (*C*), and IC2 (*D*) cells were treated with IL-4 (50 ng/ml) (+) or control buffer (−) for 10 min, and the p21-bound GDP and GTP were analyzed. The ratio of p21·GTP to p21·GDP plus p21·GTP is shown as a percentage (mean ± SEM, $n = 3$ or 4).

5–6 in all lines. In PT18 cells, the percentage of p21·GTP is relatively high in quiescent cells, and reaches ≈40% after stimulation with IL-3.

IL-4 was originally characterized as a B-cell growth factor, and it displays many other biological actions on T cells or myeloid cells. The mast cell line MC9-4 (gift of N. Harada) can grow in the presence of IL-4, but the ratio of GTP-bound p21 in MC9-4 cells did not increase following the addition of IL-4 (Fig. 4*A*). This result indicates that IL-4 inputs the growth signal through a pathway that does not cause the activation of *ras* p21. HT-2, BAF3, and IC2 cells have receptors for IL-4, and IL-4 stimulates DNA synthesis in these cells, although their viability was maintained for only a limited period. We also examined the involvement of *ras* in the signal-transduction pathway from IL-4 in these cell lines. No increase of p21·GTP induced by IL-4 was observed in any of these cells (Fig. 4 *B–D*), although IL-2, IL-3, or GM-CSF was able to stimulate the formation of p21·GTP.

Finally we tested GM-CSF, a lymphokine that stimulates the formation of colonies composed of neutrophil, macrophage, and eosinophil lineages. We added GM-CSF to two mast cell lines, IC2 and PT18, and measured the amounts of p21-bound GDP/GTP. An increase of p21·GTP was observed in both cell lines in response to GM-CSF (Fig. 5), though the magnitude of the increase was somewhat low in the case of PT18 cells. As described above, in PT18 cells the basal level of p21·GTP is high, and IL-3 activates *ras* p21 more effectively than GM-CSF.

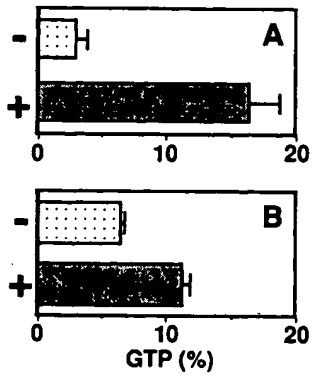

FIG. 5. Increase of p21·GTP in mast cells induced by GM-CSF. IC2 (*A*) and PT18 (*B*) cells were treated with GM-CSF (5 ng/ml) (+) or control buffer (−) for 10 min, and the p21-bound GDP and GTP were analyzed. The ratio of p21·GTP to p21·GDP plus p21·GTP is shown as a percentage (mean ± SEM, $n = 3$–5).

Table 1. Involvement of *ras* p21 in signal transduction from lymphokines

		Increase of *ras* p21·GTP			
Cell line	Cell type	IL-2	IL-3	IL-4	GM-CSF
CTLL	T cell	++			
HT-2	T cell	++		−	
BAF3	B cell		++	−	
IC2	Mast cell		++	−	++
PT18	Mast cell		++		+
MC9-4	Mast cell			−	

−, No significant increase; +, <3-fold ($P < 0.01$); ++, >3-fold ($P < 0.01$).

The results described above are summarized in Table 1. IL-2, IL-3, and GM-CSF activate *ras* p21 in every cell line we have examined regardless of cell type. In contrast, IL-4 does not activate *ras* p21 in any of the tested cells.

Effects of EGF, PMA, and Calcium Ionophore on *ras* p21 in IC2-E Cells. Activated tyrosine kinases have been shown to stimulate the formation of p21·GTP complex in fibroblast cell lines (4–6). For example, receptors for EGF have tyrosine kinase activity, and it has been shown that the binding of EGF to its receptor causes the activation of *ras* p21. To understand the relationship between the activation of *ras* p21 by IL-3 and tyrosine kinases, we compared the effects of several factors that induce proliferation or differentiation of IC2-E, a cell line derived from IC2 by stable transfection with EGF receptor cDNA. IL-3 stimulates long-term growth of IC2-E cells, but EGF can support the survival of the cells only transiently (11). Both IL-3 and EGF can enhance the accumulation of GTP-bound p21 to a similar extent (Fig. 6). The addition of both IL-3 and EGF to IC2-E cells results in differentiation to more mature mast cells characterized by increases in intracellular granulation and histamine content (11). When IC2-E cells were stimulated with both IL-3 and EGF, activation of *ras* p21 was observed, although there was no additive or synergistic increase of p21·GTP. In the presence of PMA and calcium ionophore, IC2-E cells display phenotypes similar to those induced by IL-3 plus EGF (11). However, no significant increase in GTP-complexed p21 was observed in cells treated with both PMA and calcium ionophore was observed (Fig. 6), suggesting that, at least in this cell line, protein kinase C and calcium ions function independently of *ras* p21.

DISCUSSION

Many cytokines have been identified, and some of them have been purified and molecularly cloned. Most of them act on a variety of hematopoietic cells and have diverse biological functions (8). Therefore, the signal-transduction systems

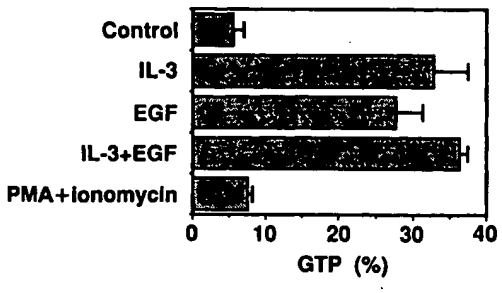

FIG. 6. Involvement of *ras* p21 in proliferation and differentiation of IC2-E cells. IC2-E cells were treated with IL-3 (5 ng/ml), EGF (5 ng/ml), IL-3 plus EGF, or PMA (100 ng/ml), plus ionomycin (200 nM) for 10 min, and the p21-bound GDP and GTP were analyzed. The ratio of p21·GTP to p21·GDP plus p21·GTP is shown as a percentage (mean ± SEM, $n = 3$–5).

from cytokines are difficult to analyze. Previous studies (4–6) have shown that various kinds of tyrosine kinase activate *ras* p21 in proliferation and transformation of fibroblast lines. In the present study, we examined whether the actions of several lymphokines lead to the activation of *ras* p21. Among T-cell-derived cytokines, IL-2 has potent growth factor activity on T cells and B cells, whereas IL-3 and GM-CSF are growth factors for various hematopoietic cells, including multipotential hematopoietic progenitors. IL-4 is a pleiotropic factor that acts on both myeloid and lymphoid cells and exhibits both growth-promoting and differentiation-inducing activities.

As summarized in Table 1, we found that IL-2, IL-3, and GM-CSF enhanced the formation of the active GTP-bound form of *ras* p21 but that IL-4 had no effect on p21. The results suggest the existence of at least two distinct pathways that cause proliferation; the results also support the idea that the accumulation of active p21·GTP induced by IL-2, IL-3, or GM-CSF is not a general consequence of the enhancement of the cell growth but rather a reflection of the regulation of *ras* p21 activity by signals from specific growth factor receptors. It must be noted that not all the stimuli that lead to cell proliferation necessarily involve p21 activation. As we have observed earlier (4), in Swiss 3T3 cells, PDGF but not bombesin plus insulin caused an increase of p21·GTP, in spite of the fact that both treatments induced the incorporation of [^{3}H]thymidine almost to the same extent.

There are several reports that introduction of a constitutively activated mutant of *ras* p21 into mast cells abrogates their IL-3 requirement (17–19). In these cases, it has been suggested that IL-3-independent proliferation is due to autocrine production of IL-3. However, in view of the present study, it may also be possible that the mutant *ras* p21 turns on signals for proliferation that are independent of the upstream signal from IL-3.

IL-2, IL-3, and GM-CSF show both low- and high-affinity binding to their receptors, and the functional receptors are thought to be composed of at least two subunits. In contrast, the receptor for IL-4 seems to bind IL-4 only with high affinity and to be a single polypeptide. From molecular cloning of cDNAs of the cytokine receptors, a number of conserved sequences have been pointed out that appear in each of the receptors for IL-2, IL-3, IL-4, and GM-CSF, though the roles of the domains have not been clarified. Striking similarity in primary structure was observed between receptors for human GM-CSF and mouse IL-3, suggesting common molecular mechanisms of signal transduction from these growth factors (20). However, it remains unsolved how the receptors activate downstream targets to turn on the signal-transduction pathways, and how they regulate the activity of *ras* p21 in a ligand-dependent manner.

Tyrosine phosphorylation has been thought to be involved in signal transduction from cytokines, including IL-2, IL-3, IL-4, and GM-CSF. A set of proteins is phosphorylated rapidly on tyrosine residues after the addition of these growth factors (21–25), though tyrosine kinases activated by the lymphokine receptors have not been identified. In contrast to the EGF receptor and the PDGF receptor, none of the cloned receptor subunits for IL-2, IL-3, IL-4, or GM-CSF have tyrosine kinase domains. Therefore, it is possible that some kinds of non-receptor-type tyrosine kinases, such as *src* family proteins, may associate with lymphokine receptors. The finding that transfection of various hematopoietic cells with oncogenes encoding tyrosine kinases, such as v-*abl* (26–30), v-*src* (30, 31), or v-*fms* (30, 32), leads to abrogation of IL-3 dependence supports the above hypothesis. We have shown that both IL-3 and EGF activated *ras* p21 in IC2-E cells, and the magnitude of the increase of active p21·GTP was similar in each case. Moreover, no additive or synergistic effects were observed between IL-3 and EGF (Fig. 6). From these results, we can suppose the involvement of tyrosine kinases in the activation of *ras* p21 induced by IL-3, and it is possible that the unknown tyrosine kinases phosphorylate common substrates with the EGF receptor and that the phosphorylated proteins may regulate *ras* p21.

It is very important to understand the mechanism of activation of *ras* p21 triggered by the lymphokines. In general, activities of signal-transducing GTP-binding proteins are regulated by two steps (33). One is the GDP/GTP exchange reaction, which increases GTP-bound active molecules (on step), and the other is the GTP hydrolysis reaction, which converts the protein-bound GTP to GDP and P_i and increases GDP-bound inactive molecules (off step). Both steps are thought to be regulated by some specific interacting molecules. In the case of *ras* p21, GTPase-activating protein (GAP) interacts with p21·GTP and enhances the GTPase activity of *ras* p21 (34, 35). Stimulators of the dissociation of the *ras* p21·GDP complex have also been reported (36–39). However, no evidence showing that these factors are responsible for altering the GDP/GTP state of *ras* p21 in response to exogenous stimuli has been presented. Several growth factor receptors and oncogene tyrosine kinases are known to phosphorylate GAP (40–43). Since these kinases also increase active p21·GTP in the cells, it is likely that phosphorylation of GAP leads to the reduction of its GTPase-stimulating activity or loss of its ability to properly interact with *ras* p21, and the GTP-bound p21 accumulates. Downward *et al.* (7) reported the activation of *ras* p21 by stimulation of the T-cell antigen receptor. According to their results, the GTPase-stimulating activity of GAP was reduced by stimulation of the antigen receptor, though they did not observe tyrosine phosphorylation of GAP.

The involvement of protein kinase C in the activation of *ras* p21 was reported in the case of T lymphocytes (7). It was reported that PMA could activate *ras* p21 in a manner similar to the stimuli of the T-cell receptor. However, p21·GTP did not increase in response to PMA and calcium ionophore in IC2-E cells (Fig. 6), suggesting distinct roles of protein kinase C between the signal-transduction pathways in T cells and mast cells.

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